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Thalidomide prolongs survival after experimental musculoskeletal injury, through an effect on mononuclear apoptosis

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ABSTRACT

Background: This study was conducted to investigate the effects of intravenous thalidomide administration in an experimental model of musculoskeletal trauma. We hypothesized that because thalidomide inhibits secretion of tumor necrosis factor alpha (TNF-α), survival of animals that received thalidomide would be significantly prolonged.

Material and methods: After an open fracture of the right femur, 24 rabbits were randomly assigned to control and thalidomide groups. Intravenous therapy with thalidomide was started 30 min after fracture. Hemodynamic monitoring of all animals was performed for 4 h. Survival was recorded and bacterial growth in blood and organs was measured after animal death or sacrifice. Blood was sampled for TNF-α measurement and for isolation of peripheral blood mononuclear cells (PBMCs). Apoptosis of PBMCs was measured by flow cytometry.

Results: Survival was significantly prolonged in the thalidomide group. Apoptosis of PBMCs was increased in the control group compared with the thalidomide group at 24 h. There were no differences in vital signs, blood and tissue cultures, and serum TNF-α concentration between the two groups.

Conclusions: Intravenous thalidomide prolonged survival in an experimental model of severe musculoskeletal injury in rabbits. Its mechanism of action did not involve TNF-α suppression but prevention of mononuclear apoptosis. In view of these promising results, further research is needed to clarify the immunomodulatory mechanism of action of thalidomide and its potential use for the management of severe trauma.

1. Introduction

The systemic inflammatory response syndrome (SIRS) is a common pathway to multiple organ dysfunction and death after severe trauma. SIRS is induced by the secretion of a variety of proinflammatory cytokines [1] and it is known that the release of mitochondrial DNA following trauma can trigger the production of proinflammatory cytokines [2].

Conflicts of Interest: None of the authors has nothing to declare related with this submission.

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Tumor necrosis factor (TNF) alpha is a central mediator of the immune response after trauma. It appears early in serum after the traumatic event, stimulates the production of other proinflammatory cytokines, primes loss of integrity of vascular endothelium, and drives apoptosis of lymphocytes [3–5]. Therefore, it may be considered a major target for therapies aiming to modulate the immune response of the host. Developed immunomodulatory strategies so far comprise monoclonal antibodies targeting TNF-α and soluble TNF receptors. These agents have been tested in phase III randomized studies of patients at severe sepsis and have failed to show survival benefit [6–10]. These results probably indicate that strategies aiming to modulate excess production of TNF-α require another approach.

Thalidomide (a-N-phthalimidoglutarimide) was initially used as a sedative and antiemetic during pregnancy, but it was withdrawn from the market due to its teratogenic effects. It is an immunomodulatory compound that selectively inhibits TNF-α synthesis [11] by reducing the half-life of messenger RNA of TNF in human monocytes [12]. Thalidomide is currently used for its anti-angiogenic and anti-TNF-α properties in the treatment of a variety of diseases like erythema nodosum leprosum, Crohn disease, cutaneous lupus erythematosus, cutaneous sarcoidosis, Behçet syndrome, graft-versus-host disease, multiple myeloma, and human immunodeficiency virus–related aphthous ulcers and wasting syndrome [13].

In a previous study by our group, thalidomide was administered orally to rats as pretreatment for experimental sepsis by *Escherichia coli* and by *Pseudomonas aeruginosa* [14,15]. Administration of thalidomide prolonged survival and reduced circulating levels of TNF-α.

The aim of this study was to investigate the effects of intravenous thalidomide administration in an experimental model of severe musculoskeletal trauma in an animal (rabbit) model. We hypothesized that because thalidomide inhibits secretion of TNF-α, survival of animals that received thalidomide would be significantly prolonged.

2. Materials and methods

2.1. Animal care

A total of 24 white New Zealand male rabbits, mean (± standard deviation [SD]) weight 3.37 ± 0.35 kg, were used for the study. The study received permission from the Veterinary Directorate of the Prefecture of Athens, according to Hellenic legislation in conformance to the 160/91 Directive Council of the EU. The study was conducted in the Center of Experimental Medicine of the ATTIKON University Hospital.

All experiments and handling were conducted under the supervision of an expert veterinary surgeon. The animals were housed in single metal cages and had access to tap water and standard balanced rabbit chow *ad libitum*. Temperature ranged between 18°C–22°C, relative humidity between 55% and 65%, and the light/dark cycle was 6 AM/6 PM.

2.2. Thalidomide preparation

Thalidomide was obtained as a white amorphous powder (Sigma, St. Louis, MO). Thalidomide is sparingly soluble in water (0.05 mg/L) and undergoes rapid spontaneous hydrolysis in the physiological pH range. Therefore, solutions of thalidomide were produced by adding 30 mg of preweighted powder to 3 mL of a (2-hydroxypropyl)-β-cyclodextrin aqueous solution (Sigma-Aldrich), as previously described [16]. The solution was further diluted to a volume of 10 mL with the addition of water for injection in an ultrasound water bath. The final 1 mg/mL thalidomide solution was prepared with the addition of another 20 mL of 5% dextrose water. Thalidomide solutions of 0.2 and 0.5 mg/mL were also produced using the same protocol.

Preliminary pharmacokinetics were done to determine the minimum IV thalidomide dose that would provide serum concentrations of 4 μg/mL, as this was shown to inhibit TNF-α *in vitro* [11,12]. In addition, the safety of the prepared thalidomide solutions for intravenous administration was determined. More precisely, 30 mL of dextrose water solutions, each containing 0.2, 0.5, and 1 mg/mL of thalidomide, were administered over 10 min to six animals. Two milliliters of blood was sampled at the end of the infusion and at 60 min after infusion. Serum was immediately separated by centrifugation at 2800 rpm for 10 min. Animals were intensively followed up for 1 wk and no adverse effects were observed.

Thalidomide concentrations in prepared solutions and animal sera were determined by a high-performance liquid chromatography (HPLC) method after protein precipitation with trichloroacetic acid, as described previously [17]. Briefly, 200 μL aliquot of thalidomide solution or serum was mixed with an equal amount of trichloroacetic acid 20% (Merck, Darmstadt, Germany) and rigorously vortexed for 30 s. The mixture was then centrifuged for 15 min at 15,000 rpm at 4°C. An aliquot of 20 μL of supernatant was injected into the HPLC system using a Zorbax Eclipse SB-C18 (4.6 × 150 mm, 5 μm) reversed-phase column (Agilent 1100 Series; Agilent Technologies, Inc; Waldbronn, Germany). The mobile phase was 10% water, 10% methanol, and 80% potassium phosphate buffer 10 mM (pH 3.0) at a flow rate of 1.5 mL/h at 37°C. A water sample treated in the same way was applied as blank. The column effluent was monitored for ultraviolet absorbency at 220 nm. The retention time of the chromatographic peak corresponding to thalidomide was 7.7 min. Concentrations of thalidomide were measured applying a standard curve generated by known concentrations of purified substance (range 0.39–100 mg/L). All determinations were performed in duplicate and their mean was applied. The interday variation of the assay was 11.5%. Although this interday variation may appear excessive, it is a result of the limitations of the assay and is similar to what was previously reported [10,18].

HPLC analysis showed that all prepared solutions were stable. Animals receiving the 0.2 mg/mL solution had mean (±SD) serum concentration 1.44 ± 0.93 μg/mL at the end of infusion; it declined to 0.64 ± 0.89 μg/mL at 60-min post-infusion. Animals administering the 0.5 mg/mL solution had mean (±SD) serum concentration 1.80 ± 0.40 μg/mL at the end of infusion; it declined to 0.91 ± 1.13 μg/mL at 60-min post-infusion. Animals receiving the 1.0 mg/mL solution had mean (±SD) serum concentration 7.30 ± 1.50 μg/mL at the end of infusion; it declined to 5.82 ± 1.45 μg/mL at 60-min post-infusion. Based on these results, the 1 mg/mL solution was selected for administration in the rest of the study because target serum levels exceeding 4 μg/mL were achieved with it.

2.3. Experimental design

Animals were initially sedated by the intramuscular injection of 25 mg/kg of ketamine and 5 mg/kg of xylazine. Anesthesia was maintained by the intramuscular administration of 15 mg/kg of xylazine for a total of 4 h. Through a midline neck incision, the left common carotid artery was identified and catheterized by a 20-gauge catheter that was stabilized by a 3-0 silk suture. The catheter was connected to a multichannel monitor (Electronics for Medicine, Houston, TX) and hemodynamic data including systolic, diastolic, and mean arterial pressure, and heart rate were recorded every 15 min for 4 h.

Resuscitation of animals was achieved by the continuous intravenous infusion of 0.9% normal saline by a catheter inserted under aseptic conditions into the right ear vein. Electric clippers were used to depilate the lateral portion of the right thigh. The right femur was then prepared using povidone-iodine, and the animals were subjected to musculoskeletal trauma using a previously described technique [19]. In brief, through a 3-cm longitudinal incision on the lateral aspect of the right thigh, the fascia lata and the fibers of the vastus lateralis were transected down to the femur. Subsequently, a comminuted fracture was created in the middle third of the right femur using a Liston bone cutting forceps. The wound was then closed in layers. To minimize animal suffering, rabbits were administered paracetamol suppositories.

Animals were then randomly assigned into two study groups, by the closed envelope technique, as follows:

- Control group ($n = 14$) that was administered 30 mL of 5% dextrose solution within 15 min, starting 30 min after the femoral fracture and
- Thalidomide group ($n = 10$) that was administered 30 mL of the 1 mg/mL prepared thalidomide solution in 5% dextrose within 15 min, starting 30 min after the femoral fracture

Five milliliters of blood was sampled after puncture under aseptic conditions of the left ear vein at time 0 and at 2, 4, 24, and 48 h. Two milliliters of blood was collected into heparinized syringes for the isolation of mononuclear cells; another 3 mL was sampled with a nonheparinized syringe; 0.5 mL was added to blood culture flasks and the remaining to pyrogen-free tubes. After centrifugation, serum was kept refrigerated at −70°C until assayed.

After operation, animals were transported to their cages and followed up daily for a total of 14 d. Surviving animals were killed at 14 d by bolus intravenous administration of sodium thiopental. Tissue samples from all dead animals were harvested aseptically, through a midline abdominal incision. Segments of 0.3–0.5 g of liver, spleen, and lower lobe of the right lung were excised using separate blades, put into separate sterile containers, and applied for quantitative culture.

2.4. Assay for TNF-α

TNF-α was measured by a bioassay on the L929 fibrosarcoma cell line, as described previously [20]. Briefly, confluent cells were washed thoroughly with Hanks solution and harvested with 0.25% trypsin/0.02% ethylenediamine tetraacetic acid (Biochrom AG, Berlin, Germany). Cells were centrifuged, resuspended in RPMI-1640 supplemented with 10% fetal bovine serum and 2 mM of glutamine (Biochrom AG), and distributed into a 96-well cell culture plate at a density of 1×10^5 cells per well. The final volume of fluid into each well was 0.05 mL. After incubation for 2 h at 37°C at 5% CO_2, 0.06 mL of serum or of standard dilutions of known concentrations of human TNF-α (range 5.75–375.00 pg/mL; Sigma) were added into each well followed by 0.05 mL of a 0.3 mg/mL dilution of cycloheximide (Sigma) to inhibit *de novo* protein biosynthesis. Incubation continued overnight; then, the supernatant of each well was discarded by aspiration and 0.1 mL of a 0.5 mg/mL methylene blue solution in methanol 99% was added. After 10 min, the dye was removed and the wells were washed thoroughly three times with 0.9% sodium chloride. The wells were left to dry and remnants of the dye in each well became soluble by the addition of 0.1 mL of 50% glacial acetic acid (Merck). Optical density in each well was read at 495 nm (Hitachi Spectrophotometer; Hitachi, Tokyo, Japan) against blank wells and control wells without added serum. Concentrations of TNF-α were estimated by the reduction of the optical density of control wells by unknown samples applying a standard curve generated by standard concentrations. All determinations were performed in quadruplicate. The inter-day variation of the assay was 13.75%, similar to what was previously reported [20].

2.5. Blood and tissue cultures

Volumes of 0.5 mL of blood were added into blood culture flasks with 20 mL of thioglycolate medium (Becton Dickinson, Cockeysville, MD) and incubated at 35°C in an automatic incubator for a total period of 7 d. Then, 1 mL was plated onto McConkey agar and identification of colonies was performed by the API20E and the API20NE systems (bioMérieux, Paris, France).

Tissue processing was carried out on the same day after sampling. Tissue segments were weighted and homogenized; a 0.1 mL aliquot was then diluted 1:10 into sterile sodium chloride four consecutive times. Another aliquot of 0.1 mL of each dilution was plated onto McConkey agar and incubated at 35°C for a total period of 3 d. Plates were incubated at 35°C and the number of viable colonies were counted into each dilution and multiplied by the appropriate dilution factor. Identification of colonies was performed as above. The lower detection limit was 10 colony-forming units/g. The number of viable cells was expressed as its $\log_{10}$ value in colony-forming units per gram.

2.6. Assay for mononuclear apoptosis

For the isolation of blood mononuclear cells, heparinized venous blood was layered over Ficoll Hypaque (Biochrom AG) and centrifuged. The separated mononuclear cells were washed three times with phosphate-buffered saline (pH 7.2). Peripheral blood mononuclear cells (PBMCs) were incubated for 15 min in the dark with the addition of the protein ANNEXIN-V conjugated to the fluorocolour fluorescein isothiocyanate (emission 520 nm; Immunotech, Marseille, France) and propidium iodide (emission 550 nm; Immunotech). Cells

staining positive for ANNEXIN-V and negative for propidium iodide after running through the EPICS XL/MSL flow cytometer (Beckman Coulter Co., Miami, FL) were considered apoptotic.

2.7. Studied parameters

Survival of animals in the two study groups was calculated at the end of the experiment. In addition, systolic, diastolic, and mean arterial pressures and heart rate of animals were recorded and analyzed. Quantitative analysis of tissue cultures obtained after animal death was also conducted.

The concentrations of circulating TNF-α and the PBMC apoptotic rate at baseline and at 2, 4, 24, and 48 h after injury were measured and compared between the two study groups.

2.8. Statistical analysis

Results were represented by their mean ± standard error. Comparisons between the two groups were performed by analysis of variance for vital signs of animals; other parameters were compared by the Mann-Whitney *U*-test. Survival of each group was estimated by Kaplan-Meier analysis; comparisons between the two groups were performed by the logrank test. Any *P* value <0.05 was considered significant.

3. Results

Survival of animals in the thalidomide group was considerably prolonged compared with animals in the control group (logrank = 5.543, $P = 0.019$) (Fig. 1).

Clarification of the possible mechanism of action of thalidomide was focused on an effect in (1) animal hemodynamics; (2) tissue bacterial growth; (3) circulating TNF-α; and (4) apoptosis of circulating mononuclear cells.

No differences were found between thalidomide and control groups regarding systolic, diastolic, and mean arterial pressures and heart rate (Fig. 2) probably showing that thalidomide was not acting on animal hemodynamics.

In a similar fashion, all blood cultures in both groups were negative for bacterial growth and quantitative tissue cultures of spleen, liver, and lung did not differ between groups (Fig. 3). Bacterial isolates were species of enterobacteriaceae.

Circulating concentrations of TNF-α at baseline and at 2 and 4 h after injury did not differ between the two groups (Fig. 4). Measurements of TNF-α concentrations at 24 and 48 h in the control group were not possible due to insufficient blood samples due to shock; therefore, no comparisons could be made at these time points.

The apoptotic rate of PBMCs between the two groups was compared. It became obvious that PBMC apoptosis was increased in the control group compared with the thalidomide group (Fig. 5).

4. Discussion

To our knowledge, this is the first study to evaluate the use of thalidomide in a posttraumatic SIRS model. Oral thalidomide has been previously used in a rat burn model; decreased plasma levels of interleukin (IL)-1 and TNF-α were demonstrated compared with the control group [21]. In one model of surgical trauma in mice, intraperitoneal thalidomide administration induced production of IL-2 and reduced synthesis of IL-10; administration attenuated the observed posttraumatic predominance of Th2 cytokines and normalized the Th1/Th2 balance [22].

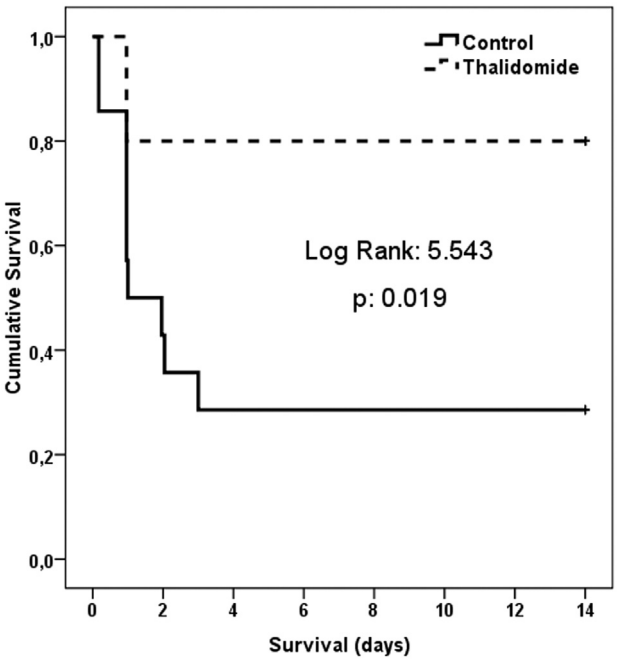

Fig. 1 – Survival curves of control and thalidomide groups. Control group animals were subjected to open femur fracture and IV administration of 30 mL of 5% dextrose water. Thalidomide group animals were subjected to open femur fracture and IV administration of 30 mL of 1 mg/mL thalidomide solution.

This is the first experimental study using intravenous thalidomide in a model of severe injury and demonstrating a favorable response in overall outcome. The pharmacodynamics of thalidomide has been evaluated thoroughly. Although the anti-inflammatory, immunomodulating, and anti-angiogenic properties of thalidomide have been described, the mechanism of action of thalidomide is not yet completely understood and results from *in vitro*, experimental, and clinical studies have been conflicting. Thalidomide is known to inhibit TNF-α via expedited degradation of messenger RNA. *In vitro* thalidomide inhibits TNF-α production from activated monocytes [11] and many studies corroborate this action *in vivo*. Thalidomide also inhibits production IL-12 [23] and IL-6 [24] from PBMCs and primes T-lymphocytes for increased production of IL-2 and IFN-γ [25]. In the clinical setting, a single oral dose of thalidomide caused a Th1-type response in healthy humans increasing the capacity of PBMCs to secrete IFN-γ [26]. Other studies indicate that thalidomide induces *in vitro* Th2 responses through increase of IL-4 levels and inhibition of IFN-γ [27], whereas clinical studies have demonstrated failure of thalidomide to contain

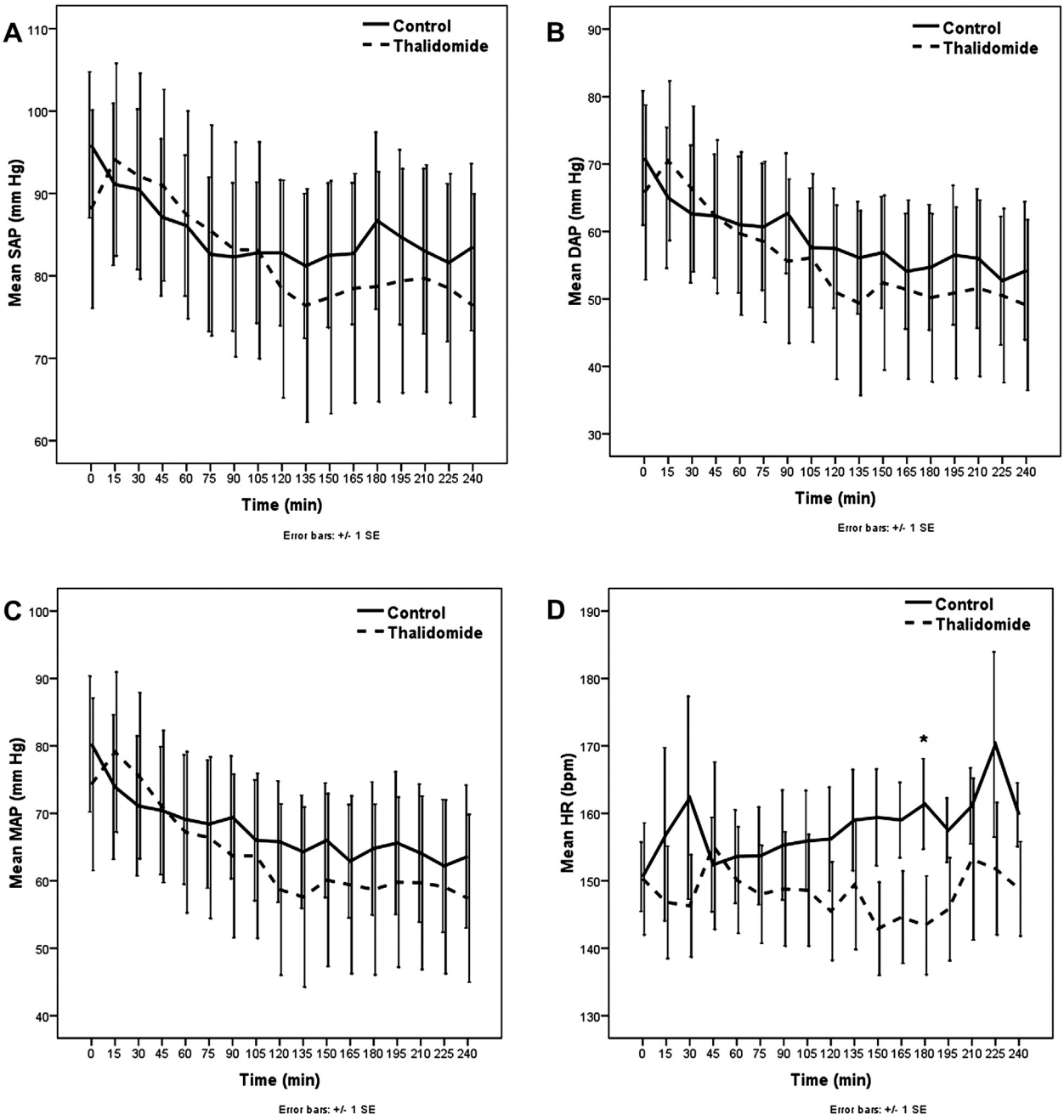

Fig. 2 – Vital signs of control and thalidomide groups over the first 4 h after injury. (A) Systolic arterial pressure (SAP), (B) diastolic arterial pressure (DAP), (C) mean arterial pressure (MAP), and (D) heart rate (HR). Results are expressed as mean; bars represent ± 1 SE. *P = 0.041.

elevated TNF-α responses within the patients with autoimmune disorders [28,29].

In a recent *in vitro* study, thalidomide was found to have a proapoptotic effect on human monocytes. The authors attributed, at least partly, the anti-inflammatory action of the drug and the observed decrease in proinflammatory cytokine levels (TNF-α, IL-6, and IL-12) after treatment with thalidomide, to this effect [30]. Probably, some of the contradictory effects of thalidomide may be explained by evidence that activity of thalidomide depends on the type of applied stimulus [31].

Our results demonstrate that IV thalidomide increased survival in rabbits in a model of severe musculoskeletal trauma. Its effect was not mediated through either hemodynamic stabilization or prevention of bacterial translocation or attenuation of circulating TNF-α. This is in accordance with results from a recent clinical study on healthy human volunteers pretreated with oral thalidomide before lipopolysaccharide (LPS) challenge. The authors observed suppression of IL-6 but no effect on TNF-α and IL-8 [32].

We aimed to determine TNF levels at 24 and 48 h in all animals and compare between the two groups at these time

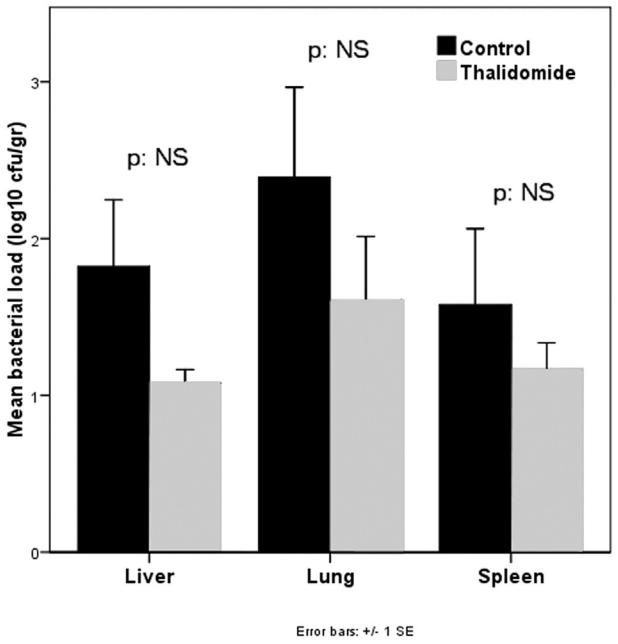

Fig. 3 – Tissue culture results of control and thalidomide groups. Bacterial loads of liver, lung, and spleen specimen cultures in log_{10} colony-forming units per gram. Results are expressed as mean; bars represent ± 1 SE.

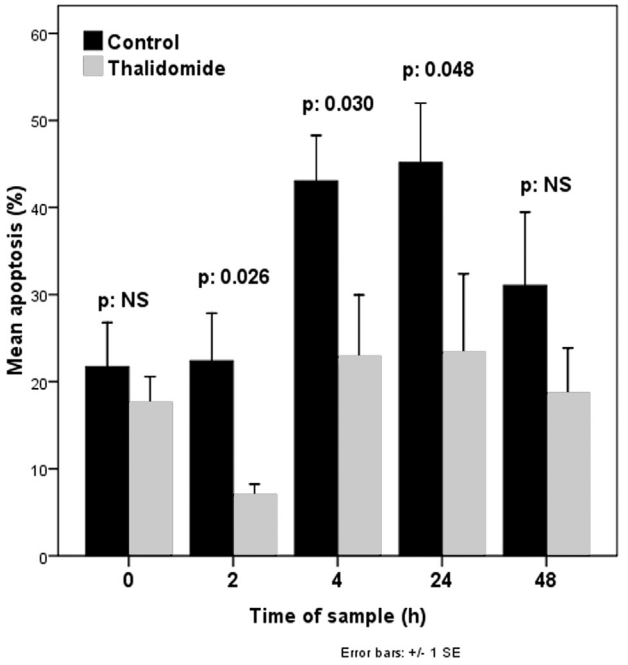

Fig. 5 – Apoptosis of mononuclear cells of control and thalidomide groups. Apoptosis of PBMCs at 0, 2, 4, 24, and 48 h after injury. Results are expressed as mean; bars represent ± 1 SE.

points. However, this proved impossible due to the increased mortality in the control group. We believe that the increased lethality of this trauma model in untreated subjects supports the efficacy of thalidomide in prolonging survival of traumatized animals. It is known that TNF-α appears early after trauma and has a very short half-life in the plasma. Consequently, if there were any differences in TNF-α levels between the control and thalidomide groups, these would have been evident at 2 and 4 h. Because there was no difference found at these time points, we concluded that thalidomide acted not by inhibiting TNF-α.

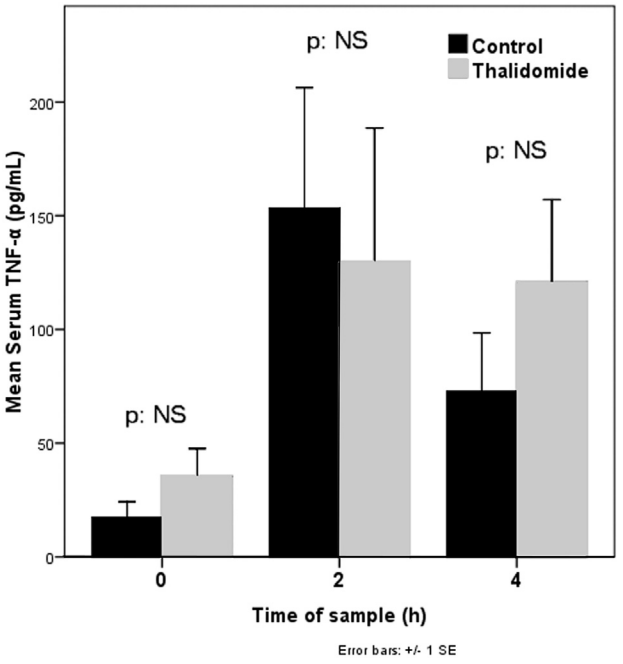

Fig. 4 – Serum TNF-α concentration of control and thalidomide groups. Comparisons between the two groups at 0, 2, and 4 h after injury. Results are expressed as mean; bars represent ± 1 SE.

Despite the fact that thalidomide did not suppress TNF-α, an immunomodulatory action of thalidomide was evident causing decreased apoptosis of PBMCs compared with the control group. Increased apoptosis of thymus, spleen, intestine, lung, and liver as well as in circulating lymphocytes and monocytes have been shown in experimental studies of multiple injuries [33,34]. This is also the case for patients at severe sepsis and for patients bearing multiple injuries [35]. The anti-apoptotic effect of thalidomide on monocytes and lymphocytes seems to be independent from its anti-TNF action. It is possible that a proapoptotic factor other than TNF-α appears early after severe trauma and that this factor may be modulated by thalidomide.

Limitations of this experimental study merit to be mentioned. Firstl, because this is an animal study, its results should be interpreted with caution especially if thalidomide is to be used in similar human studies. Second, we did not perform a power analysis or sample size determination at the outset of the study and this is inevitably a protocol flaw. However, the number of subjects is similar to what is used in many similar animal studies using rabbits [19,20,36].

In conclusion, intravenous thalidomide prolonged survival in an experimental model of severe musculoskeletal injury in rabbits. Its mechanism of action did not involve TNF-α suppression but prevention of mononuclear apoptosis. In view of

these promising results, further research is needed to clarify the immunomodulatory mechanism of action of thalidomide and its potential use for the management of severe trauma.

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